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Lytophilippines A–C: novel macrolactones from the Red Sea hydroid *Lytocarpus philippinus*

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Abstract—Lytophilippines A–C, new chloro-containing macrolactones, were isolated from the Red Sea hydroid *Lytocarpus philippinus* and their structures were elucidated by IR, UV, MS, ^{1}H and ^{13}C NMR, and by chemical degradation. The compounds gave positive results in the crown gall tumor inhibition test and brine shrimp toxicity assay.

1. Introduction

Marine natural products are produced by marine invertebrates, microorganisms and plants that have stimulated interdisciplinary studies by chemists and biologists for marine drug discovery.[1–3] The Red Sea is a unique habitat, which is characterized by extreme changes in environmental conditions such as oxygen availability, salinity, and temperature.[4] Organisms, which inhabit the Red Sea, are excellently adapted to environmental stress and offer an enormous potential as a natural source of novel biologically active molecules.[5,6]

The Hydrozoans (hydroids) are mostly all bottom dwelling animals, in a polypoid shape, and can easily be mistaken for plants. Stinging hydroids such as *Lytocarpus philippinus* in the Indo-Pacific area and Red Sea,[7] have harmless-looking, feather-like plumes that can inflict a rather nasty sting on the softer areas of human skin.[8] Hydroids are an interesting group of marine invertebrates for chemical studies but only the following papers have been published: Hydrallmanol A, a diphenyl-*p*-menthane derivative, has been isolated from the marine hydroid *Hydrallmania falcate*.[9] Four Mediterranean hydroids contain uncommon Δ^5C_{26}-sterols, but cholesterol was the principal compound.[10] The polyhydroxylated steroid, cholest-4-en-4,16β,18,22*R*-tetrol-3-one 16,18-diacetate, was found in the hydroid *Eudendrium* sp.[11] A series of Δ^5-C_{26-29} sterols, and the corresponding stanols have also been identified from the hydroid *Dynamena pumila*.[12] Four biologically active polyhalogenomonoterpenes have been isolated from four species of marine hydroids.[13] Trimethyl amine oxide, dimethyl amine and choline chloride have been isolated from the marine hydroid *Tubularia larynx*.[14]

In the course of our investigation of the chemical composition of marine invertebrates[6,15–17] we have examined one hydroid *Lytocarpus philippinus* from the Red Sea, collected in the Gulf of Aqaba, Eilat, Israel. Three novel macrolide derivatives, with unusual multi-branched chain, have been isolated from the extract. According to the literature data, we could not find any natural products isolated from this sea organism. Here we report the structure elucidation of these new chloro-containing compounds, based mainly on their spectral characteristics.

2. Results and discussion

The fireweed (hydroid) *Lytocarpus philippinus* Kirchen. (Order Hydroida, Family Plumulariidae) was collected in the Red Sea, Aqaba Gulf, Eilat, Israel. The fireweed was extracted with butanol and the extract was separated on Sephadex LH-20. The fractions were further purified by RP-HPLC to give three compounds lytophilippines A–C (**1**,**10**,**11**, see Fig. 1), which were identified by their IR, UV,

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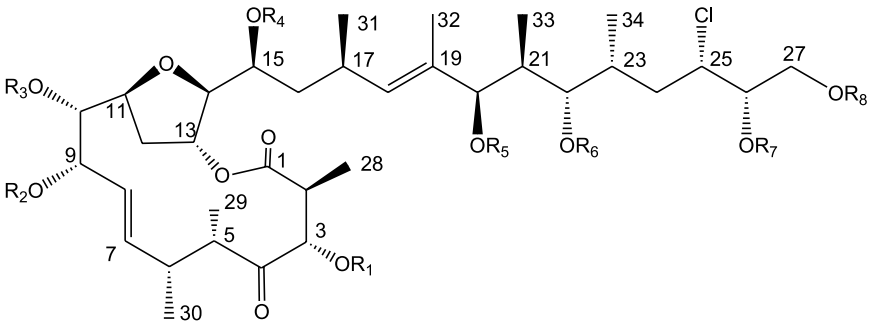

1 $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = R_8 = H$
2 $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = H$, R_8 = Piv
3 $R_1 = R_4 = R_7 = H$, R_8 = Piv, $R_2,R_3 = R_5,R_6$ =
4 $R_1 = R_4 = R_7$ = Me, R_8 = Piv, $R_2,R_3 = R_5,R_6$ =
5 $R_1 = R_4 = R_7$ = Me, R_8 = Piv, $R_5 = R_6 = H$, R_2,R_3 =
6 $R_1 = R_4 = R_7$ = Me, R_8 = Piv, $R_5 = R_6$ = p-ClBz, R_2,R_3 =
7 $R_1 = R_4 = R_7$ = (*R*)-MTPA, R_8 = Piv, $R_2,R_3 = R_5,R_6$ =
8 $R_1 = R_4 = R_7$ = (*S*)-MTPA, R_8 = Piv, $R_2,R_3 = R_5,R_6$ =
10 $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = H$, R_8 =
11 $R_1 = R_2 = R_3 = R_4 = R_5 = R_6 = R_7 = H$, R_8 =

Figure 1. Lytophilippine A–C (**1**, **10**, **11**), a new chlorinated macrolide from the Red Sea hydroid *Lytocarpus philippinus* and its derivatives **2**–**8**.

MS, and ^{1}H and ^{13}C NMR spectroscopic data and by chemical degradation.

The molecular formula of lytophilippine A (**1**) was established as $C_{34}H_{57}{}^{35}ClO_{12}$ from the observation of a pseudomolecular ion in the HRFABMS (715.3751, $[M+Na]^+$) consistent with both the ^{13}C and ^{1}H NMR spectra. The presence of seven hydroxymethine groups (broad absorption at 3490 cm^{-1} in IR spectrum), one oxymethylene group, a saturated ester or lactone (δ_C 174.5; IR 1735 cm^{-1}), and one di- and one trisubstituted double bond were evident from the IR and NMR spectra. Further, ^{1}H and ^{13}C NMR data (Table 1) disclosed the existence of a ketone, 20 methines (three of them bearing two double bonds), four methylenes, and seven methyls (one of them attached to an olefin). Since four out of six unsaturations were accounted, lytophilippine A (**1**) was inferred to contain two rings and suggesting the possibility of a highly oxygenated macrolactone.

Detailed analyses of the ^{1}H–^{1}H COSY spectrum disclosed three proton networks from H-2 to H-3 and H-28, from H-5 to H-17 (including H-31), and from H-20 to H-27 (Fig. 2). Eight hydroxyl protons and their corresponding methines (and one methylene) were also clearly coupled in ^{1}H–^{1}H COSY spectra. Long-range couplings were observed between H-2 (δ 2.53) and C-1 (δ 174.5), between H_2-13 (δ 5.10) and C-l, and between H-2 and C-3 (δ 84.0). From these connectivities and chemical shifts, the existence of unsaturated 14-membered lactone was revealed.

HMBC correlations of H-2/H-3 and H-5/H-17 to a ketone carbonyl carbon (δ 211.3, C-5) suggested that C-3 and C-5 were linked through the ketone carbonyl. The existence of an ester linkage between C-1 and C-13 was implied by HMBC correlations of H_3-28 and H_2-13 to C-1 (δ 174.5). A series of alternating oxymethines and high field methylene or methine groups were characterized in structure. At one end of the structure (C-25), the methine carbon was considerably shielded (δ 53.0) compared to the other oxymethines carbons, consistent with its presence as a halogen atom. Also a vinyl proton at δ 5.05 (C-18) was vicinally coupled to a methine at δ 2.81 (C-17) and allylically coupled to a vinyl methyl group at δ 1.60 (H-32). An alternation of oxymethines and high field methylene groups was characterized in structure as well.

The geometry of disubstituted olefin at $\Delta^{9,10}$ was assigned as *E* by ^{1}H–^{1}H coupling constants ($J_{H\text{-}9/H\text{-}10}$ = 15.2 Hz), while *E*-geometry of a trisubstituted olefin at $\Delta^{18,19}$ was revealed by NOESY cross-peaks for H-17/H_3-32 and H-18/H-20. Thus, the gross structure of lytophilippine A was elucidated to be **1**.

The relative stereochemistry of **1** was elucidated by chemical (Fig. 3) and spectral means. The compound **1**

Table 1. ^{1}H and ^{13}C NMR data of lytophilippine A (**1**)

No.	^{1}H	^{13}C
1	—	174.5
2	2.53 (1H, qd, J=7.0, 8.8 Hz)	38.5
3	4.39 (1H, dd, J=8.8, 1.7 Hz)	84.0
4	—	211.3
5	2.84 (1H, dqd, J=7.8, 6.9, 1.7 Hz)	44.9
6	2.48 (1H, dqdd, J=7.8, 6.0, 9.4, 3.0 Hz)	33.8
7	5.55 (1H, ddd, J=9.4, 15.2, 2.0 Hz)	133.4
8	5.38 (1H, ddd, J=15.2, 5.7, 3.0 Hz)	130.6
9	4.12 (1H, ddd, J=5.7, 15.2, 2.0 Hz)	72.4
10	3.74 (1H, dd, J=15.2, 4.5 Hz)	78.0
11	3.95 (1H, dtd, J=4.5, 8.0, 1.5 Hz)	69.8
12a	2.11 (1H, dt, J=13.8, 8.0 Hz)	29.8
12b	1.87 (1H, dt, J=13.8, 8.0 Hz)	
13	5.10 (1H, tdd, J=8.0, 6.6, 1.5 Hz)	69.6
14	3.66 (1H, dd, J=9.0, 6.6 Hz)	81.1
15	4.02 (1H, ddd, J=9.0, 5.0, 10.0 Hz)	66.3
16a	1.39 (1H, ddd, J=5.0, 4.9, 14.0 Hz)	39.2
16b	1.22 (1H, ddd, J=10.0, 8.9, 14.0 Hz)	
17	2.81 (1H, ddqd, J=8.9, 4.9, 6.8, 9.6 Hz)	27.9
18	5.05 (1H, brd, J=9.6 Hz)	134.3
19	—	131.7
20	4.45 (1H, d, J=1.8 Hz)	72.5
21	2.57 (1H, dqd, J=1.8, 6.8, 9.7 Hz)	41.6
22	4.00 (1H, dd, J=9.7, 2.6 Hz)	74.8
23	1.84 (1H, qddd, J=6.5, 2.6, 2.1, 10.6 Hz)	38.4
24a	1.88 (1H, ddd, J=2.1, 4.7, 13.6 Hz)	32.6
24b	1.92 (1H, ddd, J=10.6, 8.8, 13.6 Hz)	
25	4.24 (1H, ddd, J=4.7, 8.8, 5.9 Hz)	53.0
26	4.22 (1H, ddd, J=5.9, 2.4, 7.1 Hz)	80.3
27a	4.01 (1H, dd, J=12.0, 2.4 Hz)	65.5
27b	4.26 (1H, dd, J=12.0, 7.1 Hz)	
28	1.31 (3H, d, J=7.0 Hz)	8.5
29	0.94 (3H, d, J=6.9 Hz)	11.6
30	1.07 (3H, d, J=6.0 Hz)	17.1
31	0.99 (3H, d, J=6.8 Hz)	20.7
32	1.60 (3H, s)	11.5
33	0.98 (3H, d, J=6.8 Hz)	10.4
34	1.08 (3H, d, J=6.5 Hz)	15.7

was converted to pivaloyl ester (**2**), protecting the primary hydroxy group. Further reaction with 2,2-dimethoxypropane gave *O*-isopropylidene derivative (**3**). The ^{1}H NMR spectrum (see Table 2) and FABMS data (*m*/*z* 857 and 859 $[M+H]^{+}$) of **3** indicated that **3** was a di-*O*-isopropylidene derivative of **2**. The positions of three free hydroxyls in **3** were determined by the chemical shifts of oxymethine protons [δ 4.39 (H-3), 4.02 (H-15), and 4.27 (H-26)] assigned by COSY experiments, revealing that **3** is the 9,10:20,22-di-*O*-isopropylidene derivative of **2**.

The relative stereochemistry at C-2 and C-3 was determined as *threo* based on the NOESY spectrum of methine H-2 and

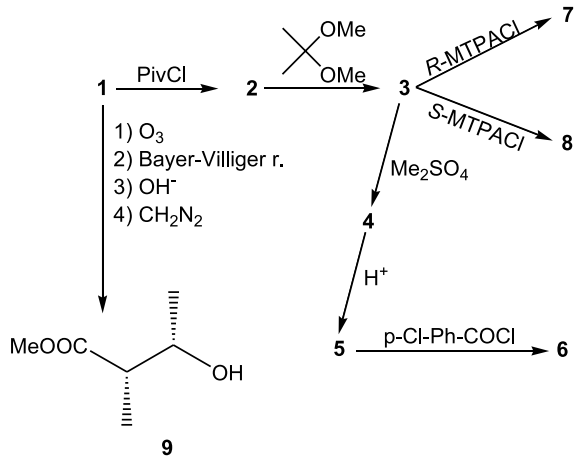

Figure 3. Reaction scheme for the preparation of the lytophilippine A derivatives **2**–**9**.

H-3 protons, since NOESY cross-peak was clearly observed for H-3/H_3-28. Further, the relative stereochemistry at C-5/C-6 was determined from vicinal $^{3}J_{H\text{-}5/H\text{-}6}$, which was approximately 8 Hz and the torsion angle corresponds to *threo* configuration and thence it follows that relative configuration is 2*S**,3*S**,5*S**,6*R**.

The relative stereochemistry of C9/C10 in **3** was determined to be *syn* based on NOESY correlations and vicinal coupling constants of **3**, while the stereochemistry of substituents about the tetrahydrofuran ring was determined using ROESY and selective 1D NOE experiments. Through space interactions were observed between H-11 and H-14, H-11 and H-12b, H-12b and H-13, and H-13 and H-14 (Fig. 4).

The relative configurations at C-14, C-15, and C-17 were elucidated on the basis of ^{1}H–^{1}H and ^{1}H–^{13}C coupling constants in addition to NOESY correlations. For the C-14/C-15 bond (Fig. 5), the $^{3}J_{H\text{-}14,H\text{-}15}$ 6.5 Hz suggested that this bond underwent a conformational change between *anti* and *gauche*. Furthermore, the $^{2}J_{C\text{-}15,H\text{-}14}$ showed a medium value 2.0 Hz, also implying that H-14 had both *anti* and *gauche* relations to OH-15 due to rotation of the bond. The values for $^{3}J_{C\text{-}16,H\text{-}17} \sim 0$ Hz obtained from the hetero half-filtered TOCSY spectrum indicated that H-14 and H-15 were *gauche* to C-16. Of the six possible rotamers arising from *erythro* and *threo*, only one pair of *threo* relationship satisfied all of these coupling data. NOESY correlations also

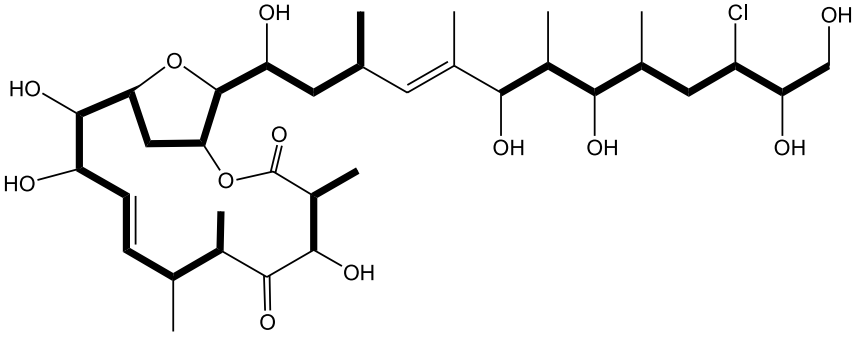

Figure 2. The ^{1}H–^{1}H COSY correlations of lytophilippine A.

Table 2. ^{1}H and ^{13}C NMR data of compound **3**

No.	^{1}H	^{13}C
1	—	174.5
2	2.53 (1H, qd, J=7.0, 8.8 Hz)	38.5
3	4.39 (1H, dd, J=8.8, 1.7 Hz)	84.0
4	—	211.3
5	2.84 (1H, dqd, J=7.8, 6.9, 1.7 Hz)	44.9
6	2.48 (1H, dqdd, J=7.8, 6.0, 9.4, 3.0 Hz)	33.8
7	5.55 (1H, ddd, J=9.4, 15.2, 2.0 Hz)	133.4
8	5.41 (1H, ddd, J=15.2, 5.7, 3.0 Hz)	130.2
9	4.52 (1H, ddd, J=5.7, 15.2, 2.0 Hz)	78.2
10	4.24 (1H, dd, J=15.2, 4.5 Hz)	82.3
11	4.06 (1H, dtd, J=4.5, 8.0, 1.5 Hz)	67.7
12a	2.13 (1H, dt, J=13.8, 8.0 Hz)	30.1
12b	1.87 (1H, dt, J=13.8, 8.0 Hz)	
13	5.10 (1H, tdd, J=8.0, 6.6, 1.5 Hz)	69.6
14	3.66 (1H, dd, J=9.0, 6.6 Hz)	81.1
15	4.02 (1H, ddd, J=9.0, 5.0, 10.0 Hz)	66.3
16a	1.39 (1H, ddd, J=5.0, 4.9, 14.0 Hz)	39.2
16b	1.22 (1H, ddd, J=10.0, 8.9, 14.0 Hz)	
17	2.81 (1H, ddqd, J=8.9, 4.9, 6.8, 9.6 Hz)	27.9
18	5.05 (1H, brd, J=9.6 Hz)	134.3
19	—	131.7
20	4.72 (1H, d, J=1.8 Hz)	73.2
21	2.63 (1H, dqd, J=1.8, 6.8, 9.7 Hz)	39.9
22	4.38 (1H, dd, J=9.7, 2.6 Hz)	75.3
23	1.89 (1H, qddd, J=6.5, 2.6, 2.1, 10.6 Hz)	37.3
24a	1.88 (1H, ddd, J=2.1, 4.7, 13.6 Hz)	32.6
24b	1.92 (1H, ddd, J=10.6, 8.8, 13.6 Hz)	
25	4.24 (1H, ddd, J=4.7, 8.8, 5.9 Hz)	53.2
26	4.27 (1H, ddd, J=5.9, 2.4, 7.1 Hz)	83.6
27a	4.43 (1H, dd, J=12.0, 2.4 Hz)	69.4
27b	4.61 (1H, dd, J=12.0, 7.1 Hz)	
28	1.31 (3H, d, J=7.0 Hz)	8.5
29	0.94 (3H, d, J=6.9 Hz)	11.6
30	1.07 (3H, d, J=6.0 Hz)	17.1
31	0.99 (3H, d, J=6.8 Hz)	20.7
32	1.60 (3H, s)	11.7
33	0.98 (3H, d, J=6.8 Hz)	10.9
34	1.08 (3H, d, J=6.5 Hz)	16.0
35	—	107.2
36	1.37 (3H, s)	26.1
37	1.39 (3H, s)	26.1
38	—	100.3
39	1.41 (3H, s)	24.6
40	1.36 (3H, s)	24.8
41	—	174.1
42	—	39.7
43	1.24 (3H, s)	23.8
44	1.24 (3H, s)	23.8
45	1.24 (3H, s)	23.8

supported the *threo* relation for the C-14/C-15 bond. For the C-15/C-16 bond, $^{3}J_{\text{H-15,H-16b}}$ was a typical value 2.2 Hz for *gauche* relationships, while $^{3}J_{\text{H-15,H-16a}}$ 10.0 Hz was indicative of an *anti* relation for H-15/H-16a. Combination of two-bond ^{1}H–^{13}C coupling constants of C-15/H-16a (5.0 Hz) and C-15/H-16b (~0 Hz) with NOESY correlations for H-14/H-16a and H-14/H-16b suggested that H-16a was

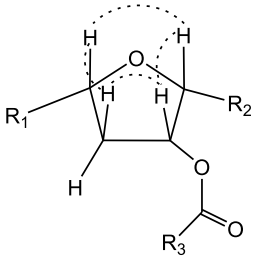

Figure 4. Key NOE correlations for protons on the tetrahydrofuran ring.

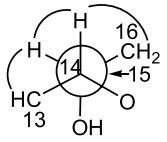

Figure 5. Rotation model for C-14–C-15 bond of lytophilippine A. NOESY correlations are illustrated by continuous curve.

gauche to C-14 and 12-OR and that H-16b was *gauche* to C-14 and *anti* to 12-OR. Therefore, the 1,3-chiral center of C-15/C-17 was elucidated to have a *syn* relation and relative configuration is 9*S**, 10*S**, 11*S**, 13*R**, 14*S**, 15*S**, 17*R**.

The relative configuration at C-20/C-22 was established as 20,22-*anti* by Rychnovsky's method,[18] because the two acetonide methyl carbon atoms and one ketal carbon appeared at δ 24.6, 24.8, and 100.3 ppm, respectively, in the ^{13}C NMR spectrum of **3**. The *anti*-acetonide exists in the twist boat conformation owing to the 1,3-diaxial interaction. The vicinal coupling constants $J_{20,21}$=1.8 Hz and $J_{21,22}$=9.7 Hz in the ^{1}H NMR spectrum of **3** indicate that the three substituents at C-20, C-21, and C-22 are *threo* and *erythro* disposed, respectively. The relative configuration at C-22/C-23 was determined by the vicinal coupling constant $J_{22,23}$=2.6 Hz. This value showed that the substituents were 22,23-*threo*. From these results, the relative configurations of **3** were determined to be 20*R**, 21*S**, 22*S**, and 23*R**.

J-based configuration analysis, a powerful method recently developed by Murata[19] for the elucidation of relative stereochemistry in acyclic structures using $^{3}J_{\text{H,H}}$ values was successfully applied to our molecule. Using ^{1}H NMR homonuclear and heteronuclear *J* values of the functionalized portions C-25/C-26 were successfully determined (Table 2) and evaluated. For the 1,2-methine systems along C25–C26, the coupling constant data were consistent and sufficient to determine their relative configurations. In fact, for *threo* rotamer, the hydrogens linked to the two gauche carbons should come within the range of NOE, while in the case of C/C-*anti* conformation (*erythro* rotamer), no NOE should be observed between them. In our case, NOE experiments revealed spatial proximity for H-25 and H-26 and we identified the right rotamers along C25/C26 as *threo* and the configuration of the fragment C25/C26 must be 25*S*, 26*S* or its enantiomer (25*R*, 26*R*).

Absolute configuration at C-5 and C-6 was determined after ozonolysis and following Bayer–Villiger reaction. Lytophilippine A (**1**) was treated by ozone and subsequent Baeyer–Villiger reaction was performed by treatment of the crude mixture of **1** with trifluoroperoxyacetic acid. The mixture was hydrolysed and treated with an excess of diazomethane. As result of the all these reactions, the methyl ester of nilic acid was isolated and give $[\alpha]_{D}$=+30.2, which agree with methyl (2*S*,3*S*)-2-methyl-3-hydroxy butyrate (**9**) (lit.[20,21] for this compound give $[\alpha]_{D}$=+36.8 or +27.8, respectively).

Further, this methyl ester (**9**) was also compared with *S*,*S* enantiomer, synthesized by means of chiral gas–liquid chromatography (see Experimental). From retention times it is evident that both carbons, C-5 and C-6 have the *S*,*S* absolute configurations.

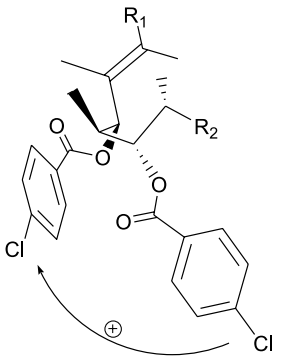

Figure 6. Stereostructure of *p*-chlorbenzoates (**6**) of lytophilippine A (**1**). Positive chirality between the two chromophores (two *p*-chlorbenzoate groups) of **6**.

The compound **3** was methylated and permethylated derivative (**4**) was hydrolyzed at heating in methanol with acetic acid according to Sviridov et al.[22] The major product (**5**), i.e. the compound with a five membered acetonide was reacted with *p*-chlorbenzoyl chloride afforded the 20,22-bis-*p*-chlorobenzoate (**6**). Consequently, the CD exciton chirality method[23] was applied and the CD spectrum of **6** showed a large positive first Cotton effect at 253 nm ($\Delta\varepsilon$ +8.6) and a second negative Cotton effect at 228 nm ($\Delta\varepsilon$ −7.1), indicating that the absolute configurations of **1** were 20*R*,22*S*, respectively, see Figure 6.

To determine the full stereochemistry of the remaining isolated asymmetric centers at C-3, C-15 and C-26, compound **3** was treated with *R*(−) and *S*(+) MTPA chloride in pyridine solution[24] at room temperature for 2 h to give ester derivatives **7** [from *R*(−)MTPA] and **8** [from *S*(+)-MTPA] (Fig. 7), respectively, that were believed to be appropriate for the application of the Mosher method,[24] because of the absence of mutual influence between the three introduced MTPA groups. ^{1}H NMR resonances of the esters **7** and **8** were assigned by an extensive analysis of 1D and 2D NMR spectra. Significant $\Delta\delta$ values ($\delta_{S\text{-MTPA-ester}} - \delta_{R\text{-MTPA-ester}}$) for the protons near to the derivatized chiral centers C-3, C-15, and C-26 were observed. Inspection of the molecular models of the MTPA esters **7** and **8** indicated that there is no steric hindrance to all the MTPA groups adopting the 'ideal conformation' having trifluoromethyl, ester carbonyl, and carbinol methine proton coplanar. These results enabled the absolute configurations at C-3, C-15, and C-26 in **1** to be determined as *S*, *S*, and *S*, respectively. The results from MM2 calculations and molecular modeling, together with those described above, indicated that the most stable conformation of **1** is depicted in Figure 8, i.e. the final absolute stereochemistry is 2*S*, 3*S*, 5*S*, 6*R*, 7*E*, 9*S*, 10*S*, 11*S*, 13*R*, 14*S*, 15*S*, 17*R*, 18*E*, 20*S*, 21*S*, 22*S*, 23*R*, 25*S*, and 26*S*.

The structure of lytophilippine B (**10**) was elucidated to be the same as that of lytophilippine A except that long chain acyl was inserted in the molecule of lytophilippine A. The structure was easily assigned to a saturated fatty acyl chain; however, NMR analysis alone was not able to establish its chain length. This was determined by catalyzed transesterification in HCl–MeOH and analysis of the resultant methyl ester by GC–MS. A single peak was observed which was identical in retention time and fragmentation pattern with methyl palmitate.

The distinctive ^{1}H and ^{13}C shifts of C-27 versus those at C-26, C-22, C-20, etc. (Table 3) indicated that an ester group was attached at this position. A HMBC correlation between the H-27 proton and ester carbonyl at (C-1′) δ 173.7 confirmed this assignment. HMBC correlations from H-2′ to C-1′, C-3′ and the overlapped methylene envelope at ~30 ppm additionally confirmed the presence of this fatty acyl chain. In the acyl moiety, the key signals relative to

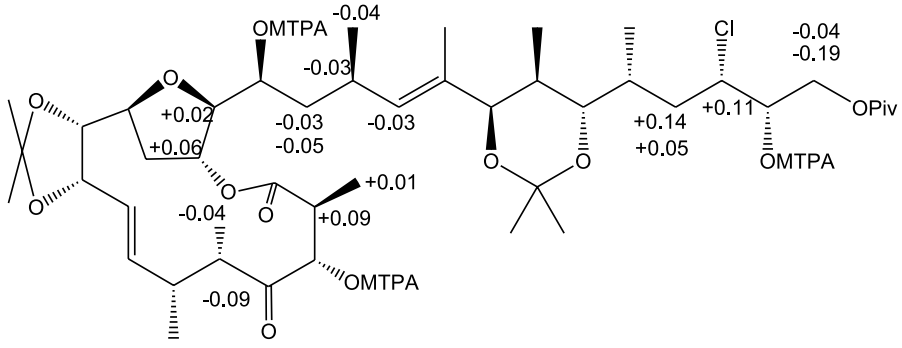

Figure 7. $\Delta\delta=(\delta_S-\delta_R)$ values (ppm) obtained for the MTPA esters.

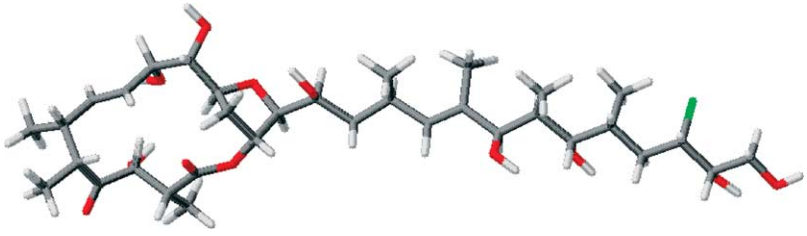

Figure 8. 3D-MM2 model of compound **1**.

Table 3. ^{1}H and ^{13}C NMR spectroscopic data of compound **10** and **11**

No.	^{1}H of **10**	^{1}H of **11**	^{13}C of **10**	^{13}C of **11**
1′	—	—	173.7	173.8
2′	2.24 (2H, t, J=7.3 Hz)	2.10 (2H, t, J=7.5 Hz)	26.5	26.5
3′	1.53 (2H, m)	1.43 (2H, m)	30.3	30.3
4′–7′	1.32 (8H, m)	1.30 (8H, m)	30.3	30.3
8′, 11′	1.32 (4H, m)	1.96 (4H, m)	30.3	27.3
9′, 10′	1.32 (4H, m)	5.39 (2H, m)	30.3	131.7
12′–15′	1.32 (8H, m)	1.30 (8H, m)	30.3	30.3
16′	1.32 (2H, m)	1.30 (2H, m)	32.5	32.5
17′	1.23 (2H, m)	1.23 (2H, m)	23.1	23.1
18′	0.88 (3H, t, J=7.0 Hz)	0.88 (3H, t, J=7.0 Hz)	14.0	14.0
27	5.32 (2H, q, J=6.5 Hz)	5.32 (2H, q, J=6.5 Hz)	70.4	70.4

protons located at the ends of the alkyl chain clearly indicated its unbranched nature. The structure of compound **10** is 27-O-palmitate of lytophilippine A.

The NMR spectra of lytophilippine C (**11**) indicated that the structure of this compound is very similar to **10**, apart from for the presence of the chemical shifts at δ 5.39 (^{1}H NMR) and 131.7 (^{13}C NMR) in the long acyl chain. Thus, the structure of lytophilippine C was elucidated to be the same as that of lytophilippine B except that one ethylenylidene (C_2H_2=26 mass units) was inserted in the hexadecyl side chain of lytophilippine B. The geometrical configuration of the double bond was assumed to be *Z* from the *J* value (7.2 Hz) of the neighboring methy1ene,[25] although a spin–spin coupling was not observed between olefinic protons. The fragmentation of the *Z*-alkenyl side chain in lytophilippine C appeared to resemble that of N-mono-unsaturated fatty acyl pyrro1idide.[26,27] The structure of **11** is 27-O-oleate of lytophilippine A.

The marine dinoflagellates of the genus *Amphidinium* are a rich source of a series of polyketide macrolides known as the amphidinolides,[28–30] which possess cytotoxic properties. These compounds are very similar to the lytophilippines A–C, which are presented in this paper.

The presence of Cl or Br in the macrolides causes significant changes in the physico-chemical characteristics, increasing their reactivity and demonstrated antibacterial, antiviral and other activities.[31] According to these suggestions were by us discovered new chloro-containing 15-membered macrolides, namely lytophilippines A–C **1**–**3**, which show a modest activity against different microorganisms (see Table 4). They are active against *Escherichia coli*, but inactive against the Gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*.

Table 4. Bioactivities of lytophilippines A–C (**1**, **10**, **11**)

Test organism	**1**	**2**	**3**
Staphylococcus aureus[a]	0	0	0
Bacillus subtilis[a]	0	0	0
Escherichia coli[a]	26.3	20.4	19.5
Saccharomyces cerevisiae[a]	0	0	0
Artemia salina[b,c]	3.2	6.4	4.8
Agrobacterium tumefaciens[c,d]	28±3[e]	68±6	65±7

[a] Samples (10 μg) were applied on 50.8 mm paper disks, values are diameters (mm) of inhibitory zones.
[b] In μg/mL (minimum lethal doses).
[c] The details in Section 3.
[d] Presented values are means of three determinations.
[e] Percentage of crown gall tumor inhibition (±SD).

The crown gall tumor inhibition test has been used for the active antitumor agents produced in vivo by organisms and is also used to evaluate extracts for different pharmacological activities. The isolated compounds were evaluated by their ability to inhibit the growth of crown gall tumors on potato discs inoculated with *Agrobacterium tumefaciens* carrying a tumor-inducing plasmid. All compounds showed significant inhibition of the growth of crown gall tumors on potato disks, suggestive of in vivo antitumor activity. All the extracts assayed demonstrated crown gall tumor inhibition, ranging from 32% for compound **2** to 72% for compound **1**.

The second test, i.e. brine shrimp lethality test showed that all three compounds were active against *Artemia salina*. As has been proposed,[31] macrolide have an allelopathic role in hydroids. Their potent cytotoxicity could play a role in hydroids defence against marine organisms, particularly active predators: tropical fishes, starfishes, sea urchins and/or nudibranchs. It is known that hydroid species as well as ascidians or sponges are conspicuous members of marine fouling and benthic communities. Their soft-bodied morphology provides hydroids with little obvious structural defense from predation. No information able about hydroid's defence compounds, but it is know that marine sponges and/or acsidians have been a rich source of natural products, and the ecological roles of these metabolites have been investigated in a few studies only.[32,33]

3. Experimental

3.1. General experimental procedures

UV–VIS spectra were measured in MeOH within the range of 220–550 nm in a Cary 118 (Varian) apparatus. A Perkin–Elmer (Perkin–Elmer, Norwalk, CT, USA) model 1310 IR spectrophotometer was used for scanning IR spectroscopy as neat films. Circular dichroism (CD) measurement was carried out under dry N_2 on a Jasco-500A spectropolarimeter at 24 °C. A Perkin–Elmer Model 1310 (Perkin–Elmer, Norwalk, CT, USA) IR spectroscope was used. NMR spectra were recorded on a Bruker AMX 500 spectrometer (Bruker Analytik, Karlsruhe, Germany) at 500.1 MHz (^{1}H), 125.7 MHz (^{13}C). High- and also low-resolution MS were recorded using a VG 7070E-HF spectrometer (70 eV). HRFABMS (positive ion mode)

were obtained with a PEG-400 matrix. GC–MS of the fatty acid methyl esters were done using a Finnigan 1020 B (Finnigan MAT, San Jose, CA, USA) single-state quadrupole GC–MS instrument in the EI mode. Gas chromatography analysis was in a Hewlett Packard HP 5980 gas chromatograph (Hewlett Packard, Czech Republic).

3.2. Chromatography of fatty acid derivatives

The fatty acid composition was determined by GC–MS after transmethylation with 5% HCl in methanol. The fatty acid methyl esters and pyrrolidides were chromatographed by a fused silica capillary column of chemically bonded liquid phase (Supelcowax 10, 0.2 mm ID, 60 m length, Supelco) and helium carrier gas at a flow rate of 0.35 mL min^{-1}. The column temperature was programmed from 50 °C held for 1 min, to 100 °C at a rate of 10 °C min^{-1}, and then raised to the final hold temperature of 270 °C at a rate of 5 °C min^{-1}. The mass spectra of methyl esters and pyrro1idide agreed with previously published data.[26,27]

3.3. Chiral chromatography

FS capillary column HYDRODEX β-3P ID 0.25 mm, length 25 m, with the stationary phase [heptakis-(2,6-di-*O*-metyl-3-*O*-pentyl)-β-cyclodextrine] from Macherey-Nagel GmbH & Co. KG, Düren, Germany was used. Oven temperature: 50–150 °C at 2 °C/min, then to 240 °C at 5 °C/min, carrier gas helium, 20 cm/s, detector FID, 300 °C, injection of 1 μL mixture in methylene chloride (for standards: containing 0.5 mg/mL of each analyte), split (100:1), 300 °C.

3.4. Animal materials

The fireweed (hydroid) *Lytocarpus philippinus* Kirch. (Order Hydroida, Family Plumulariidae) was collected by hand from rocks (from 10 to 15 m deep), on 31 June 2003 in the Red Sea, Aqaba Gulf, Eilat, Israel. The voucher specimens are deposited in the collection of the third author (V.M. Dembitsky). Fresh hydroid was put into ethanol and stored at −10 °C under nitrogen.

3.5. Isolation

Hydroid was extracted three times by butanol and the extracts were further chromatographed by means of the Sephadex LH-20 column with chloroform–methanol 7:3 and then separated by RP-HPLC on a Discovery C18 column (Supelco) particle size 5 μm, length×I.D. (250 mm×21.2 mm) using a linear gradient from 20% H_2O and 80% acetonitrile to 1% water and 99% acetonitrile over 25 min, with a flow rate of 9.9 mL/min and monitored by a variable wavelength detector at 208 nm was used to separate of compounds **1** (28.9 mg), **10** (4.1 mg) and **11** (3.5 mg) in the crude extract.

3.5.1. Pivaloyl ester (2). Lytophilippine A (11.5 mg) was dissolved in pyridine (0.5 mL), and pivaloyl chloride (12 μL) was added. After 15 min the reaction solvents were evaporated to dryness. The residue was subjected to TLC (benzene–ethyl acetate, 1:1) to yield 10.7 mg of pivaloyl ester **2**. HRFABMS calcd. for $C_{39}H_{65}{}^{35}ClO_{13}$ $[M+Na]^+$ 799.4011, found 799.4016.

3.5.2. Bis-9,10:20,22-*O*-isopropylidene derivative (3). Compound **2** (10.5 mg) was treated with dimethoxypropane (0.3 mL) and pyridinium *p*-toluenesulfonate (0.3 mL) in CH_2Cl_2 (2.0 mL) at room temperature for 12 h. After evaporation the solvent, the residue was purified on a silica gel TLC (hexane–EtOAc, 6:11) to give diacetonide **3** (yield 9.2 mg). HRFABMS calcd for $C_{45}H_{73}{}^{35}ClO_{13}$ $[M+Na]^+$ 879.4637, found 879.4641; ^1H and ^{13}C NMR data see Table 2.

3.5.3. Methyl ether (4). To a stirred solution of 1 mL of methyl triflate in 2 mL of di-*tert*-butylpyridine was added 7.0 mg of acetonide (**3**) in 2 mL of chloroform. A condenser was then fixed to the flask, and the solution was brought to reflux. After 15 h of stirring, the solution was allowed to cool and 0.5 mL of concentrated ammonium hydroxide was added. After a further 2 h of stirring, the mixture was poured into water and extracted with dichloromethane. The combined organic layers were then washed with three 5 mL portions of 10% hydrochloric acid. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The resulting oil could be purified by chromatography eluting with 15% ethyl actate in hexanes to give the methyl ether **4** as clear oil, yield 6.4 mg. HRFABMS calcd for $C_{48}H_{79}{}^{35}ClO_{13}$ $[M+Na]^+$ 921.5423, found 921.5418.

3.5.4. 9,10-*O*-Isopropylidene derivative (5). To the solution of compound **4** (6.2 mg) in 1 mL of methanol was added 250 μL of acetic acid and mixture was kept at 40 °C 2 h. The solution was cooled and 0.5 mL of concentrated of sodium hydrogen carbonate was added, the mixture was poured into water and extracted with dichloromethane. The organic layer was dried (Na_2SO_4) and concentrated under reduced pressure. The resulting oil could be purified by chromatography eluting with 15% ethyl acetate in hexanes to give 3.3 mg of the acetonide (**5**). HRFABMS calcd for $C_{45}H_{75}{}^{35}ClO_{13}$ $[M+Na]^+$: 881.4793, found 881.4800.

3.5.5. *p*-Chlorobenzoate (6). Acetonide **5** (3.1 mg) was dissolved in pyridine (1.0 mL), and 4-chlorobenzoyl chloride (20 μL) and a catalytic amount of 4-dimethylaminopyridine were added. After 18 h methanol (2.0 mL) and hexane (1 mL) were added, and the reaction solvents were evaporated to dryness. The residue was subjected to TLC (benzene–EtOAc, 9:1) to yield **6** (yield 3.7 mg). HRFABMS calcd for $C_{59}H_{81}{}^{35}Cl_3O_{15}$ $[M+Na]^+$ 1157.4540, found 1157.4544; CD (EtOH) λ_{ext} ($\Delta\varepsilon$) 253 nm (+8.6), 236 (~0), 228 (−7.1).

3.5.6. (*S*)-MTPA Esters (7). To a stirred solution of ~1.0 mg of the hydroxy compound (**1**) in 0.3 mL dry pyridine was added 20 μL of (−)-MTPA chloride. The mixture was stirred under N_2 at room temperature for 1 h and the solvent was then removed by blowing with N_2. The residue was redissolved in 2 mL of EtOAc–hexane and filtered through a Sep-Pak silica column. After removing the solvent under vacuum, the residue was separated by RP-HPLC (ODS column, 100% acetonitrile) to yield ~1.0 mg of *S* ester as a colorless gum. HRFABMS calcd for $C_{75}H_{94}{}^{35}ClF_9O_{19}$ $[M+Na]^+$ 1527.6148, found 1527.6141; ^1H NMR data, see Figure 7.

3.5.7. (*R*)-MTPA Esters (8). Prepared as described for *S* esters. An amount of ~1.0 mg of compound (**1**) and 20 μL of (+)-MTPA chloride gave 0.9 mg of *R* esters as a colorless gum. HRFABMS calcd for $C_{75}H_{94}{}^{35}ClF_9O_{17}$ $[M+Na]^+$ 1495.5932, found 1495.5936.

3.5.8. Methyl 3-hydroxy-2-methyl-butyrate (9). A stream of 4% ozone was passed through a solution of the mixture lytophilippine A (**1**, 15 mg) in dichloromethane (0.5 mL) at −78 °C for 5 min. The solution was flushed with nitrogen and concentrated. The residue was dissolved in CH_2Cl_2 (0.2 mL), and treated with TFPA prepared by adding trifluoroacetic anhydride (0.1 mL) to a 30% aqueous H_2O_2 (0.1 mL) in CH_2Cl_2 (10 mL) at 4 °C for 12 h and evaporated. After gentle heating, the mixture was heated under reflux for 70 min. The mixture was concentrated and the residue was dissolved in methanol (0.5 mL), hydrolyzed for 10 min at 60 °C with 1 mL of 1 M KOH, cooled and acidified to pH 2.5 and treated with excess diazomethane in ether. The resulting solution was distilled (up to 90 °C/10 mmHg) to give **9** with $[\alpha]_D^{23}=+30.2°$. The distillate was also further separated by chiral GC. Mass spectrum was identical with synthesized methyl ester (**9**), see bellow. Retention time was 32.83 min.

3.5.9. Methyl (2*S*,3*S*)-3-hydroxy-2-methyl-butyrate (9). This compound was synthesized from 260 mg of methyl (3*S*)-3-hydroxybutyrate (from Sigma-Aldrich) according to Frater et al.[34] The product was distilled at 77 °C and 10 mmHg (77–78/10 mmHg)[34], yield 180 mg (62%). The literature[34] describe yield 67.8%. $[\alpha]_D^{22}=+29.3$ (MeOH; $c=0.54$), lit.[34,35] give $[\alpha]_D^{22}=+19.1$ and/or +27.8, respectively; IR ν_{max}: 3420, 3020, 1725, 1460 cm^{-1}; ^1H NMR ($CDCl_3$): δ 4.03 (1H, m), 3.67 (3H, s), 2.55 (1H, m), 1.21 (3H, d), 1.18 (3H, d). ^{13}C NMR ($CDCl_3$): δ 175.3 (C-1), 68.9 (C-3), 51.2 (OCH_3), 46.5 (C-2), 19.8 (C-4), 11.3 (2-Me); EI-MS: *m/z* 132 $[M]^+$, 114 $[M-H_2O]^+$, 101 $[M-OCH_3]^+$, 88 $[M-CH_3CHO]^+$. The chiral chromatography showed four peaks, one major (93.8%) with retention time 32.87 min and three minor (1.2, 1.9 and 3.1%, respectively).

3.6. Transesterification and preparation of pyrrolidide

The fatty acyl components were obtained as their methyl esters by reaction of the lytophilippines B and C with methanolic HCl followed by column chromatography including elution with *n*-hexane–diethyl ether (9:1).

The methyl oleate was dissolved in freshly distilled pyrrolidine (0.5 mL), acetic acid (0.1 mL) was added, and the mixture was heated at 100 °C for 1 h. Excess pyrrolidine was blown off in a stream of nitrogen at 50 °C, and then the residue was taken up in hexane–diethyl ether (1:1, v/v; 2 mL) and was washed three times with water (1 mL portions). After drying over anhydrous sodium sulfate, the product was injected to GC–MS.

3.6.1. Lytophilippine A (1). Colorless powder (28.9 mg). $[\alpha]_D^{23}=-45.8$ (*c* 0.036, MeOH). UV λ_{max} (MeOH, nm) (log ε): 282 (2.07). IR (film, cm^{-1}): ν_{max} 3490 (OH), 2900, 1735 (C=O), 1680. HRFABMS (*m/z*): 715.3751 $[M+Na]^+$, calcd for $[C_{34}H_{57}^{35}ClNaO_{12}+Na]^+$ 715.3748; NMR spectra see Tables 1 and 2.

3.6.2. Lytophilippine B (10). Colorless powder (4.1 mg). $[\alpha]_D^{23}=-37.4$ (*c* 0.009, MeOH). UV λ_{max} (MeOH, nm) (log ε): 282 (1.98). IR (film, cm^{-1}): ν_{max} 3600 (OH), 2900, 1735 (C=O), 1680. HRFABMS (*m/z*): 953.5737 $[M+Na]^+$, calcd for $[C_{50}H_{87}^{35}ClNaO_{13}+Na]^+$ 953.5732; NMR spectra see Tables 1 and 2.

3.6.3. Lytophilippine C (11). Colorless powder (3.5 mg), $[\alpha]_D^{23}=-44.2$ (*c* 0.008, MeOH). UV λ_{max} (MeOH, nm) (log ε): 282 (2.07). IR (film, cm^{-1}): ν_{max} 3600 (OH), 2900, 1735 (C=O), 1680. HRFABMS (*m/z*): 979.5893 $[M+Na]^+$, calcd for $[C_{52}H_{89}^{35}ClNaO_{13}+Na]^+$ 979.5889; NMR spectra see Tables 1 and 2.

3.7. Antibacterial tests

The test organisms were *Bacillus subtilis*, *Staphyloccocus aureus Escherichia coli* and *Saccharomyces cerevisiae* (Czechoslovak Collection of Microorganisms, Brno). Antibacterial assays were carried out according to the literature.[36] The amounts used were 50 μg of compound per test disk (see Table 4).

3.8. Brine shrimp toxicity bioassay

The sample (~0.05 mg) was dissolved in 50 μL of DMSO and added to a test vial of artificial seawater (3.0 mL). Approximately 20 brine shrimp, *Artemia salina*, were added to the vial. The brine shrimp were observed periodically over a 24 h period. A positive assay was the death of all brine shrimp.

3.9. Crown gall tumors on potato disks test

The *Agrobacterium tumefaciens* potato disc assay for tumor/antitumor induction was performed according to the procedure described in literature.[37] The potatoes were sterilized by immersion in ethanol 70% for 2 min and in 50% sodium hypochlorite solution (active chlorine 30 g/l) for 30 min. Then, the potatoes were rinsed several times with sterilized distilled water, in the laminar flow hood. A core of tissue was extracted from each tuber with a sterilized 1.5 cm cork borer. Discs of 0.5 cm were cut with a scalpel. The potato discs were placed in 1.5% agar Petri dishes. To each potato disc was applied 0.05 mL of a solution containing 2 mL of a broth culture of *A. tumefaciens* (48 h culture of ca. 109 cells/mL), 1.5 mL of sterile H_2O and 0.5 mL of the solution test extract (8 mg of extract in 2 mL of DMSO filtered through 0.22 mm filters). Control discs were prepared with sterile DMSO instead of test extract. A minimum of three Petri dishes (5 disks/dish) ($n=15$–25) was used for each test compound and the control. Following preparation, the Petri dishes were placed in an incubator at 27 °C for 12–21 days. To determine the number of tumors, the potato discs were stained with a solution of I_2 (1 g) and KI (2 g) in 300 mL distilled H_2O. Significant activity was indicated when two independent assays gave 20% or greater inhibition.

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